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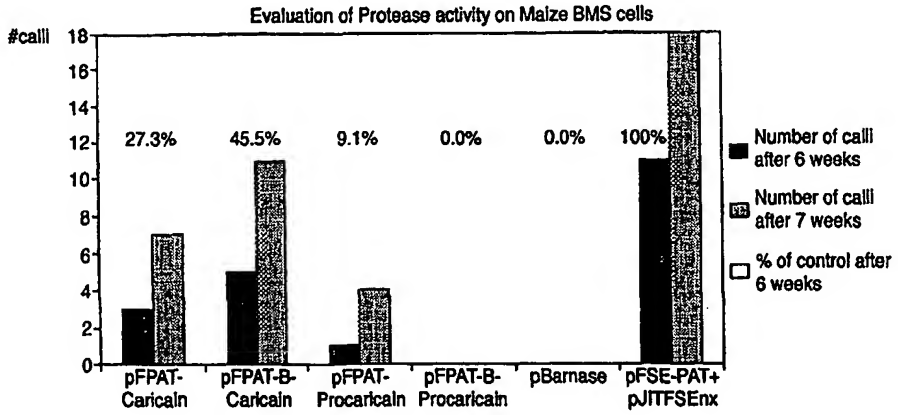

(57) Abstract

An isolated DNA construct comprising: a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism; b) a second DNA sequence comprising a DNA sequence coding for a protease enzyme operably linked and under the control of the promoter sequence specified at (a); whereby the presence or absence of the exogenous inducer or the activation of the developmental gene promoter specified at (a) results in expression of said protease enzyme. These constructs are preferably rendered reversible by the presence of further elements. They can be used in plant or mammalian cells for disruption of cell function, controlling senescence and modifying the metabolism of stored proteins.

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DNA CONSTRUCTS COMPRISING PROTEASE ENCODING SEQUENCES OR INHIBITORS THEREOF

The present invention relates to DNA constructs, for use in transformations of plant and mammalian cells. In particular, the present invention relates to a DNA construct which enables cell function to be disrupted and, optionally, for the disruption of cell function to be reversed. The present invention also relates to a DNA construct which enables the process of senescence in plants to be slowed down or inhibited.

A further DNA construct according to the present invention enables the process of protein metabolism, in particular of stored protein, to be modified in order to improve plant performance or yield. Such constructs may be used to delay the germination of seed as required to prevent pre-harvest sprouting.

The DNA constructs of the present invention therefore make use of DNA sequences which are able to inhibit proteins required for cell function, or which control senescence or germination and/or which control protein metabolism, in particular of stored protein. DNA sequences such as those encoding precursor proteins are useful in the present invention.

Proteins having a pre-pro-enzyme or pro-enzyme precursor protein are particularly useful in the present invention. Examples of proteins having such a precursor protein include protease enzymes. One such protease is a cysteine protease. Cysteine proteases (CPs) are members of a large multigene family in plants (Praekelt *et al.*, 1988; Goetting-Minesky and Mullin, 1994), animals (Wiederanders *et al.*, 1992) and protozoa (Mallinson *et al*, 1994). Cysteine proteases are synthesised as an inactive precursor (Praekelt *et al.*, 1988). The pre-pro-enzyme is targeted to the secretory pathway (Marttila *et al.*,1995) and is post-transcriptionally processed in the vacuoles by proteolytic cleavage of the propeptide fragment to produce the active enzyme (Hara-Nishimura *et al.*, 1993 and 1994).

Plant cysteine proteases participate in different metabolic events of physiological importance. During seed germination and plant senescence they are involved in protein degradation (Jones *et al.*, 1995; Valpuesta *et al.*, 1995; Smart *et al.*, 1995) and play a key role in the mobilisation of stored protein during germination (Boylan and Sussex, 1987). During seed development, cysteine proteases catalyse the post-translational processing of protein precursors into their mature form (Hara-Nishimura *et al*, 1995). In addition, some are subjected to hormonal regulation either by giberellic acid (Koehler and Ho, 1990; Watanabe

et al., 1991) or ethylene (Cervantes *et al.*, 1994; Jones *et al.*, 1995). Others are induced in response to stress like wounding (Linthorst *et al.*, 1993; Lidgett *et al.*, 1995), dehydration (Guerrero *et al.*, 1990), cold (Schaffer and Fischer, 1988) or are implicated in plant-microbe interactions (Goetting-Minesky and Mullin, 1994).

Germination specific cysteine proteases have been characterised for barley (Marttila *et al.*, 1995), rice (Watanabe *et al.*, 1991), maize (Debarros and Larkins, 1994), chick-pea (Cervantes *et al.*, 1994), vetch (Becker *et al*, 1994) and a cysteine protease has been described for oil seed rape (Comai and Harada, 1989).

Taylor *et al.*, 1995 describe the study *in vitro* of proteolytic enzymes. A number of proteases are described including members of the cyteine protease family. Recombinant proregions of papain and papaya protease IV, produced in *E. coli*, can act as as differential fast binding inhibitors of the four naturally occuring papaya cysteine proteases. It is taught that evidence suggests that the cleavable "pro" regions in protease precursors function not only to inhibit protease activity but also to assist folding. Recombinant propeptides expressed in E. coli were found to be selective inhibitors of their cognate protease in the nanomolar range, while other cysteine proteases tested were unaffected by the presence of the propeptide up to 10 micromolar (Volkel *et al.*, 1996). There is no teaching or suggestion, however, of any use for such proteases or any teaching of the DNA constructs of the present invention.

It is desirable to provide methods of controlling functions such as reversible cell disruption, senescence and stored protein metabolism in an organism.

Thus the invention provides a DNA construct comprising:-

a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism;

b) a second DNA sequence comprising a DNA sequence coding for a protease enzyme operably linked and under the control of the promoter sequence specified at (a);

whereby the presence or absence of the exogenous inducer or the activation of the developmental gene promoter specified at (a) results in expression of said protease enzyme.

The use of exogenous inducers to control promoter activity is well known. Promoters may be stimulated by a variety of factors, for example, environmental conditions, presence of a pest or pathogen or the presence of a chemical. In particular, suitable inducible promoters are induced by an exogenous chemical stimulus such that the exogenous inducer is a chemical. The external chemical stimulus is preferably an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals.

The inducible promoter most preferably comprises an inducible switch promoter system such as, for example, a two component system such as the *alcA/alcR* gene switch promoter system described in our published International Publication No. WO 93/21334, the ecdysone switch system as described in our International Publication No. WO 96/37609 or the GST promoter as described in published International Patent Application Nos. WO 90/08826 and WO 93/031294, the teachings of which are incorporated herein by reference. Such promoter systems are herein referred to as "switch promoters". The switch chemicals used in conjunction with the switch promoters are agriculturally acceptable chemicals making this system particularly useful in the method of the present invention.

Similarly, development specific promoters, are also well known. For example some promoters, such as the malate synthase (MS) promoter (see Graham et al., 1990, Plant Mol Biol. 15, 539-549, Comai et al 1992, Plant Physiol., 98, 53-61), are active during early seedling development in plants. Further examples include promoters of cysteine protease promoters themselves, such as those described in WO 97/35983. Alternatively, plant development promoters from genes in the glyoxysome such as isocitrate lyase, and promoters from genes in the the aleurone layer such as α-amylases (Baulcombe et al. (1987) Mol. & Gen Genet. 209, 33-40). Scutellum gene promoters such as that of carboxypeptidase or promoters from germin genes (Lane et al., (1991) J. Biol. Chem. 266, 10461) may also comprise promoters which are active at a particular stage in the development of plants.

Promoters active at a selected phase of development may also be isolated using conventional methods, for example by applying a reverse transcriptase polymerase chain reaction (RT-PCR) strategy to RNA from organisms at the selected development stage, and comparing these using RNA blot analysis with RNA from cells at different development stages. These sequences can then be identified and sequenced conventionally and the

promoter sequences determined. For a particular application of the present, as described hereinafter, senescence-induced promoter.

Tissue specific promoters are also known in the art or can be isolated using similar methods. Examples of known tissue specific promoters include anther- and/or tapetum-specific promoter or a pollen-specific promoter. Other such promoters can be isolated in particular by using the techniques described in International Patent Application No. WO 90/08826.

The term "protease enzyme" used herein refers to a naturally occuring protease enzyme or a fragment thereof or a variant of either of these, provided it has protease activity.

The term "variant" as used herein includes experimentally generated variants or members of a family of related naturally-occurring peptides as may be identified by molecular genetic techniques. Such techniques are described for example in US Patent No. 5,605,793, US Patent No. 5,811,238 and US Patent No 5,830,721, the content of which is incorporated herein by reference. In essence this technique involves expression of the parental gene in a microbial expression system such as *Escherichia coli*. The particular system selected must be validated and calibrated to ensure that biologically active peptides are expressed, which may be readily achieved using a *in vivo* bioassay. The gene, or preferably a collection of related genes from different species, may be subject to mutagenic polymerase chain reaction (PCR) as is known in the art. Fragmentation of the products and subsequent repair using PCR leads to a series of chimeric genes reconstructed from parental variants. These chimeras are then expressed in the microbial system which can be screened in the usual way to determine active mutants, which may then be isolated and sequenced. Reiteration of this molecular evolution DNA shuffling cycle may lead to progressive enhancement of the desired gene properties. The advantage of a technique of this nature is that it allows a wide range of different mutations, including multi-mutation block exchanges, to be produced and screened.

Other variants are those which are experimentally generated using for example the molecular evolution techniques. Preferably such variants will have improved activity or function as compared to the native sequences. Suitable improvements may be in relation to the intrinsic specific activity of the protein, or by altering a physical property such as stability.

Other variants may be identified or defined using bioinformatics systems. An example of such a system is the FASTA method of W.R. Pearson and D.J. Lipman PNAS (1988) 85:2444-2488. This method provides a rapid and easy method for comparing protein sequences and detecting levels of similarity and is a standard tool, used by molecular biologists. Such similar sequences may be obtained from natural sources, through molecular evolution or by synthetic methods and comparisons made using this method to arrive at "opt scores" which are indicative of the level of similarity between the proteins.

With these constraints in mind, a skilled person would be able to isolate other members of the family of peptides, for example by designing probes or primers based upon a naturally occurring protease enzyme but modified within the limits of the FASTA opt score range. These probes could then be used to screen libraries such as cDNA or genomic libraries using conventional methods, in order to isolate other enzymes with similar activity. Hybridisation conditions used during these screening exercises are either low or high stringency, preferably high stringency conditions as are routinely used in the art (see for example "Molecular Cloning, A Laboratory Manual" by Sambrook et al, Cold Spring Harbor Laboratory Press, N.Y.). In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as strong as 1x SSC and so on.

Once found other family members could also be subject to molecular evolution techniques or DNA shuffling as described herein, in order to improve the properties thereof. All peptides obtained in this way should be regarded as a variant.

In a second aspect, the invention provides plant germplasm comprising a DNA construct as described above.

Expression of protease enzymes in a controllable manner may be used for a variety of purposes. For example, they may be used to disrupt cell function which may find application for example in the production of male sterile plants as described for example in WO 90/08830 Preferably in such an instance, the disruption of cell function is reversible. This can be achieved in the present case at the protein level, by controllably expressing a protein which disrupts the protease enzyme.

Thus, according to a third aspect of the present invention there is provided a DNA construct comprising:-

a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism;

b) a second DNA sequence comprising a DNA sequence coding for a protease enzyme capable of disrupting cell function operably linked and under the control of the promoter sequence specified at (a);

c) a third DNA sequence comprising a promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism; and

d) a fourth DNA sequence comprising a DNA sequence, the product of which is capable of inhibiting the protein specified in (b) operably linked and under the control of the promoter sequence specified at (c),

whereby the presence or absence of the exogenous inducer or the expression of the developmental gene promoter specified at (a) enables cell function to be disrupted and whereby the presence or absence of the exogenous inducer or the expression of the developmental gene promoter specified at (c) prevents or reduces the disruption of cell function.

Examples of promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoters useful at (c) above include those defined above in relation to (b).

According to a fourth aspect of the present invention there is provided plant germplasm comprising a plant comprising a DNA construct as described above.

According to a fifth aspect of the present invention there is provided a mammalian cell comprising a DNA construct as described above.

According to a sixth aspect of the present invention there is provided the use of a DNA construct as described above to disrupt cell function and to optionally reverse said cell disruption.

Preferably, the first DNA sequence of the DNA construct capable of reversibly disrupting cell function further comprises an operator sequence, responsive to a repressor protein coded for by a DNA sequence comprised within a fifth DNA sequence which is operably linked and under the control of a sixth DNA sequence comprising an inducible promoter sequence, responsive to the presence or absence of an exogenous inducer. Examples of promoters suitable for use as the sixth DNA sequence include those listed above for use as the second DNA sequence.

Preferably, the fourth DNA sequence of the DNA construct which is capable of reversibly disrupting cell function comprises a DNA sequence coding for a member of a family of proteins which are naturally associated with the DNA sequence specified in (b). The fourth DNA sequence preferably comprises a DNA sequence coding for a protease propeptide. Alternatively, the protein is an enzyme inhibitor or even an antibody, raised against the product of the said second DNA sequence.

Preferably, the protease is a targeted or non-targeted cysteine protease enzyme. Examples of naturally occuring cysteine protease enzymes and nucleic acid sequences which encode them are described for instance in WO 97/35983.

Preferably, the developmental gene promoter is an early seedling/ seed promoter. Alternatively, it may be a pathgen-induced promoter.

Preferably, the DNA construct is capable of disrupting cell function in a plant cell or a mammalian cell.

According to a seventh aspect of the present invention there is provided a construct comprising:-

(a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of a plant; and

(b) a second DNA sequence comprising a DNA sequence, the product of which is capable of inhibiting a protein capable of controlling senescence in a plant operably linked and under the control of the promoter sequence specified at (a),

whereby the presence or absence of the exogenous inducer or the activity of the developmental gene promoter enables the synthesis of the protein capable of controlling

senescence to be down-regulated or its activity to be substantially reduced, thereby enabling the process of senescence to be slowed down.

According to an eighth aspect of the present invention there is provided the use of a DNA construct as defined above to down-regulate the synthesis or substantially reduce the activity of a protein capable of controlling senescence.

According to a nineth aspect of the invention, there is provided plant germplasm comprising a plant comprising a DNA construct according to the seventh aspect defined above.

Preferably, the second DNA sequence of the DNA construct which is capable of slowing down the process of senescence comprises a DNA sequence coding for a member of a family of proteins which are naturally-associated with the protein capable of controlling senescence. Preferably, the second DNA sequence comprises a DNA sequence coding for a protease propeptide. Alternatively, the DNA sequence codes for a protease partial sense or antisense RNA. In yet a further alternative, the DNA sequence codes for an antibody raised against the product of the second DNA sequence and capable of inhibiting the activity thereof.

Preferably, the protein capable of controlling senescence is a protease enzyme. The protease may be derived from plants, fungi, bacteria or animals or may be a fragment thereof or a variant of either of these which has protease activity. Most preferably, the protease is derived from plants, fungi, bacteria or animals.

Preferably, the protease is a targeted or non-targeted cysteine protease as described above.

Preferably, the developmental gene promoter is a senescence-induced promoter.

Preferably, the DNA construct is capable of down-regulating or substantially reducing the activity of the protein capable of controlling senescence in a plant cell.

According to a tenth aspect of the present invention there is provided a DNA construct comprising:-

(a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of a plant; and

(b) second DNA sequence comprising a DNA sequence, the product of which is capable of down-regulating or inhibiting a protein capable of controlling metabolism of stored protein in a plant operably linked and under the control of the promoter sequence specified at (a),

whereby the presence or absence of the exogenous inducer or the expression of the developmental gene promoter specified at (a) enables the synthesis of the protein capable of controlling metabolism of stored protein to be down-regulated or its activity to be substantially reduced, thereby altering the nature, the mobilisation and/or the distribution of stored products in plants.

In particular, such a construct can be used to prevent pre-harvest sprouting by causing germination of the plant to be delayed.

According to an eleventh aspect of the present invention there is provided a plant, plant seed or plant cell comprising a DNA construct as defined above in the tenth aspect.

According to a twelth aspect of the present invention there is provided the use of a DNA construct as defined above to down-regulate the synthesis or substantially reduce the activity of a protein capable of controlling metabolism of stored protein, for example as occurs during germination.

Preferably, the first DNA sequence of the DNA construct capable of controlling metabolism of stored protein further comprises an operator sequence responsive to a repressor protein coded for by a DNA sequence comprised within a third DNA sequence which is operably linked and under the control of fourth DNA sequence comprising an inducible promoter sequence responsive to the presence or absence of an exogenous inducer, whereby expression of the repressor protein prevents modification of the metabolism of stored protein.

In this way, the effects of the construct on the metabolism of stored protein can be reversed.

Alternatively or preferably additionally, the DNA construct also comprises a fifth DNA sequence comprising a DNA sequence coding for a heterologous protein which is operably linked and under the control of a sixth DNA sequence comprising an inducible promoter sequence, responsive to the presence or absence of an exogenous inducer whereby expression of the heterologous gene halts modification to stored protein metabolism.

Preferably, the protein capable of controlling metabolism of stored protein, which is capable of being inhibited by the product encoded by the DNA sequence comprised within the DNA construct in a plant, is a protease enzyme, suitably an endogenous protease enzyme. Preferably, it is a targeted or non-targeted cysteine protease enzyme as described above.

Preferably, the second DNA sequence of the DNA construct which is capable of modifying metabolism of stored protein in a plant comprises a DNA sequence coding for a product which is naturally associated with the protein capable of modifying metabolism of stored protein.

Preferably, the second DNA sequence comprises a DNA sequence coding for a protease propeptide.

Preferably, the second DNA sequence comprises a DNA sequence coding for a protease sense RNA or partial sense RNA or a DNA sequence coding for a protease antisense RNA. Alternatively, it may code for an antibody which is specific for and inhibits the protein capable of modifying metabolism of stored protein.

Proteases may be derived from plants, fungi, bacteria or animals or may comprise fragments of these or variants of either of these which have protease activity. Most preferably the protease is derived from a plant, fungi, bacteria or animal.

Preferably, the second DNA sequence comprises a DNA sequence coding for a cysteine protease sense, partial sense or antisense RNA.

Preferably, the heterologous protein is a protease.

Preferably, in this case, the developmental gene promoter is an early seedling promoter.

Preferably, the DNA construct is capable of down-regulating the synthesis or substantially reducing the activity of the protein capable of modifying metabolism of stored protein in plants.

According to a preferred embodiment of the present invention there is provided a DNA construct capable of reversibly disrupting cell function as defined above wherein the protein capable of disrupting cell function is a cysteine protease enzyme and wherein the protein capable of inhibiting said cysteine protease enzyme is a protease propeptide.

According to a further preferred embodiment of the present invention there is provided a DNA construct capable of slowing down or inhibiting senescence as defined

above wherein the protein capable of controlling senescence is a mature cysteine protease enzyme and wherein the protein capable of inhibiting said cysteine protease enzyme is a cysteine protease propeptide.

According to another preferred embodiment of the present invention there is provided a DNA construct capable of modifying metabolism of stored protein as defined above wherein the protein capable of modifying metabolism of stored protein is a cysteine protease enzyme and wherein the said cysteine protease enzyme is inhibited by a DNA sequence coding for either a full or partial antisense or partial sense RNA of said cysteine protease enzyme.

The term "DNA construct" - which is synonymous with term such as "cassette", "hybrid" and "conjugate" - includes DNA sequences directly or indirectly attached to one another , such as to form a cassette. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, intermediate each DNA sequence. The DNA sequences may furthermore be on different vectors and are therefore not necessarily located on the same vector.

The term "naturally-associated" includes complete or partial precursor proteins of the protein of interest or proteins which would bind *in vivo* to the protein of interest. These may be naturally occurring or synthetic.

The term "precursor protein" includes proteins which are formed prior to and converted into the protein of specific interest and includes pre-pro-protein and pro-protein organisations i.e. proteins comprising a mature enzyme region, a propeptide region and/ or a target region.

The term "protein" includes polypeptides comprising one or more chains of amino acids joined covalently through peptide bonds, oligopeptides comprising three or more amino acids covalently linked through peptide bonds and peptides consisting of two or more amino acids linked covalently through peptide bonds.

The term "protease" includes a pre-pro-enzyme, a pro-enzyme or a mature enzyme which are able to hydrolyse peptide bonds in proteins and peptides. Such proteases may be derived from plants, fungi, bacteria or animals. An example of a protease useful in the present invention is a cysteine protease (CP).

The term "product" includes a protein, a precursor protein and antisense or partial sense RNA to a DNA sequence capable of performing the stated function.

The DNA sequences of the present invention may be genomic DNA sequences which are in an isolated form and are, preferably, operably linked to DNA sequences with which they are not naturally associated, or the DNA may be synthetic DNA or cDNA.

The present invention also provides a genetically transformed organism such as a plant and parts thereof, such as cell protoplasts and seeds, having incorporated, preferably stably incorporated, into the genome of the organism the DNA constructs of the present invention. Thus, the present invention provides an organism the cells of which can be reversibly inhibited at an appropriate developmental stage in which the organism contains, preferably stably incorporated in its genome, the recombinant DNA construct as defined above. In this regard, the protein capable of disrupting cell function can be detargeted (i.e. to become cytosolic) or retargeted to a specific area of the cell such as to mitochondria or chloroplasts. The disruption of cell function can then be reversed by the expression of a protein which specifically inhibits the protein disrupting cell function.

An advantage of the present invention is that the protein capable of disrupting cell function is specifically inhibited by a protein encoded by a DNA sequence comprised within the DNA construct. In addition, for two of the applications, the inhibition takes place at the protein level rather than at the DNA level. Therefore any cytotoxic protein synthesized and accumulated prior the onset of repression will also be inhibited.

The present invention also provides a plant, in which the synthesis of a protein capable of controlling senescence is down-regulated by expression of a protein which specifically inhibits it.

An advantage of the DNA constructs capable of slowing down or inhibiting senescence of the present invention is that in so doing the yield of the plant in which it incorporated is increased. Further details on the regulation of senescence are given in our International Patent Application No. WO 95/07993 which is incorporated herein by reference.

The suppression of the proteins involved in senescence can be controlled by the use of either "antisense" or "partial-sense" technology.

A DNA construct according to the present invention may be an "antisense" construct generating "antisense" RNA or a "partial-sense" construct (encoding at least part of the functional gene product) generating "partial-sense" RNA.

"Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith).

"Partial-sense RNA" is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such partial-sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith) without the ability to encode a functional protein. Suitable partial-sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299).

The suppression of the proteins involved in senescence can alternatively be controlled by the use of the propeptide of a protease involved in the senescence process. Over-expressing a propeptide alone would inhibit a mature protease thus delaying senescence

Other approaches which are, or become, available may also be used.

The DNA constructs of the present invention may advantageously be used to optimise plant performance and yield.

As a route to modifying storage protein content, nature or localisation in plants we propose the selective down-regulation of proteins such as cysteine proteases throughout the plant (Mino and Inoue, 1988). This may be achieved through the use of "sense" or "antisense" technology. A beneficial effect might, therefore, be obtained through partial sense or anti-sense down-regulation of these proteases in seed, cotyledons, roots or stems. A

number of other key enzymes have also been described to play a role in storage protein mobilisation and/or translocation. The down-regulation of these genes using the same partial sense or anti-sense strategies might also be useful in the present invention to improve plant performance.

The inhibition of these proteins may also be achieved by the use of a precursor protein of the protein to be inhibited.

To prevent or reverse storage protein metabolism to be altered, the synthesis of the partial-sense or anti-sense could be repressed using a repressor/operator strategy in combination with an inducible promoter. Alternatively, an heterologous protease gene the sequence of which differs enough from the endogenous protease gene could be expressed under the control of an inducible promoter. This would avoid the occurrence of any down-regulation by the partial-sense or anti-sense.

During seed maturation and storage the seed promoter would drive, for example, partial sense to a cysteine protease, and so inhibit protein degradation and thus reduce the loss of storage proteins and the incidence of pre-harvest sprouting.

It will be appreciated that the use of the developmental gene promoter of the present invention restricts expression of the sequence to which it is operatively linked to a suitable stage of plant development, and also means that it is not necessary to continue to apply an exogenous inducer to the plant throughout its lifetime. This has both economic and ecological benefits.

Cysteine proteases are synthesised as an inactive precursor comprising a propeptide fused to the mature enzyme. The propeptide seems to inhibit the mature enzyme by folding on it. Furthermore, the propeptide alone, expressed in *E. coli* and purified, shows inhibition of the mature enzyme *in vitro* (Taylor *et al.*, 1995). Inhibition of cysteine proteases could therefore be carried out by expressing the pro-peptide under the control of an inducible or stage specific, resulting in a reduction of protein loss. The physiological effect may be reversed by down-regulating the synthesis of the pro-peptide using a repressor/operator strategy or by expressing an heterologous protease which sequence differs enough from the endogenous one to avoid any binding of the propeptide.

As a further strategy to obtain cell death/inhibition, system, re-targeted cysteine protease could be expressed, for example targeted to the mitochondria or chloroplasts. Expression of a cysteine protease in a different cellular compartment may have inhibitory results. This protease could be fused to a chloroplast or a mitochondrial targeting sequence, such as the pre-B from *Nicotiana plumbaginifolia* (Boutry et al., [1987], Nature, 328, 341), therefore achieving protein degradation in these sensitive organelles. CPs could also be retargeted to the cytoplasm or to the endoplasmic reticulum, fused or not to an ER retention signal.

Since CPs have been shown to be associated with cell autolysis in plants (Minami and Fukuda, 1995), an alternative approach would be to express cysteine proteases with no targeting sequences, so achieving cytosolic expression. Again this may cause protein degradation and cell death.

The DNA constructs of the present invention are introduced into a plant by transformation. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the DNA construct is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The DNA constructs of the present invention can therefore be used in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

We have also isolated and characterised nucleic acid sequences which code for novel cysteine proteases and these are described in our co-pending International Patent Application No. WO97/35983.

The present invention will now be described only by way of non-limiting example with reference to the accompanying drawings, in which:-

Figure 1 shows a schematic outline of the construction of maize transformation vector, pFSE-PAT

Figure 2 shows a schematic outline of the construction of vector, pFUN-β.

Figure 3 shows a schematic outline of the construction of maize transformation vector, pFUN-Caricain.

Figure 4 shows a schematic outline of the construction of maize transformation vector, pFUN-β-Caricain.

Figure 5 shows a schematic outline of the construction of maize transformation vector, pFUN-Procaricain.

Figure 6 shows a schematic outline of the construction of maize transformation vector, pFUN- β -Procaricain.

Figure 7 shows a schematic outline of the construction of maize transformation vectors, pFPAT-Caricain, pFPAT-β-Caricain, pFPAT-Procaricain and pFPAT-β-Procaricain

Figure 8 illustrates that cysteine proteases can inhibit plant cell regeneration.

Example 1 - OVER-EXPRESSION OF CPs IN CORN CALLI TO DEMONSTRATE THEIR POTENTIAL AS INHIBITORY GENES

Aim

The objective of this experiment was to show that expression of a de-targeted or re-targeted protease in cultured BMS corn cells results in a reduction of cell viability as measured by the establishment of transgenic calli following transformation, in comparison with the establishment of calli transformed with a vector not containing the inhibitory cassette,

thereby indicating that the expression of a protease in the cytosol or in the mitochondria is inhibiting cell growth. As an example we chose caricain (or proteinase omega), a cysteine protease from *Carica papaya* (EMBL: X66060). The targeting sequence of the pre-pro-enzyme was removed in order to prevent its entry to the secretory pathway. As a result the protease should be expressed in the cytoplasm as a proenzyme (pFPAT-Procaricain) or a mature enzyme (pFPAT-Caricain). In addition, the protease was re-targeted to the mitochondria, again as a proenzyme (pFPAT-β-Procaricain) or a mature enzyme (pFPAT-β-Caricain).

Construction of the maize transformation vectors

pFPAT-Caricain, pFPAT-β-Caricain, pFPAT-Procaricain and pFPAT-β-Procaricain are plant transformation vectors in which the expression of caricain partial or full length cDNA is under the control of the maize ubiquitin promoter (Ub-pro) fused to its intron (UB-int) and the terminator of the *Agrobacterium tumefaciens* nopaline synthase gene. In addition, the vectors contain a " PAT cassette", conferring resistance to the herbicide Basta to the transformed cells. A schematic outline of the construction of the maize transformation vectors pFPAT-Caricain, pFPAT-β-Caricain, pFPAT-Procaricain and pFPAT-β-Procaricain is given in Figure 1-6.

The " PAT cassette" was excised as an *Asc*I fragment from pIG-PAT and filled-in using Klenow polymerase. The generic maize transformation vector pFSE-PAT was then created by inserting the "PAT cassette" into a pFSE2 vector digested by *Sma*I and *Hind*III (to remove one of the two *Fse*I site) and filled-in using Klenow polymerase (Figure 1). Effector cassettes can be inserted using the unique *Fse*I site.

A targeting sequence for plant mitochondria was obtained from a cDNA clone encoding the β-subunit of an ATPase gene isolated from *Nicotiana plumbaginifolia* (Chaumont *et al.*, 1994). The targeting sequence fragment was obtained by a first digest of the cDNA clone with *Nco*I, a filling-in reaction using Klenow polymerase and a subsequent digest with *Bam*HI. The sequence was then ligated into the pFUN vector digested with *Kpn*I, blunt-ended using T4 DNA Polymerase and subsequently digested with *Bam*HI, to create PFUN-β (Figure 2).

The mature caricain-encoding fragment was obtained by PCR on pET-WT-Caricain using a forward oligonucleotide, MatCaric (5'GTTTATTAATGAAGATGGATCCATGCTGCCCGAGAAT3'), modified to introduce a *Bam*HI site and an optimised translation start at the 5'end of the mature sequence, and a reverse oligonucleotide, MatCaric-R (5'GTTAGCAGCCGGATCCTCAATTTTTA3'). The PCR fragment was then digested with *Bam*HI and ligated into the pFUN and PFUN-β vectors also digested with *Bam*HI, to create PFUN-Caricain (Figure 3) and PFUN-β-Caricain (Figure 4) respectively.

A procaricain-encoding fragment was obtained by a digest of pET-WT-Caricain with *Nde*I, a filling-in reaction using Klenow polymerase and a further digest with *Bam*HI. The DNA fragment was then ligated into the pFUN vector digested with *Kpn*I, blunt-ended using T4 DNA Polymerase and further digested with *Bam*HI, to create PFUN-Procaricain (Figure 5).

In addition, the procaricain-encoding fragment was obtained by simultaneous digest of pET-WT-Caricain with *Nde*I and *Bam*HI, followed by a filling in reaction using Klenow polymerase. The DNA fragment was then ligated into the PFUN-β vector digested with *Sma*I to create PFUN-β-Procaricain (Figure 6).

*Fse*I cassettes containing procaricain or mature caricain DNA driven by the maize polyubiquitin promoter (UBI) and its intron and followed by the *nos3'* terminator were excised from pFUN-Caricain, pFUN-β-Caricain, pFUN-Procaricain and pFUN-β-Procaricain. The *Fse*I cassettes were then cloned into the *Fse*I site of pFSE-PAT to generate the plant transformation vectors pFPAT-Caricain, pFPAT-β-Caricain, pFPAT-Procaricain and pFPAT-β-Procaricain respectively (Figure 7).

Transformation of BMS corn cells

pFPAT-Caricain, pFPAT-β-Caricain, pFPAT-Procaricain and pFPAT-β-Procaricain were transformed into maize BMS cells. A double control was obtained by co-transforming BMS cells with pFSE-PAT, which contains the PAT selection cassette alone, and pJITFSEnx. Vector pJITFSEnx consists of a double enhanced CAMV 35S promoter driving the GUS reporter gene and does not include any PAT selection cassette.

This combination of vectors was used as a negative control as it should not inhibit calli formation. Since cells transformed with pJITFSEnx alone will not regenerate on bialophos, it was also used as a co-transformation control. The frequency of co-transformation that can be achieved using BMS wisker transformation is important for subsequent experiments (example 2) and it was estimated by counting the proportion of regenerating calli that also express GUS (double transformants).

A positive control was provided by pBarnase which contains a PAT selection cassette followed by the potent cytotoxic ribonuclease gene barnase driven by the CAMV 35S promoter.. This construct was anticipated to strongly reduce the successful establishment of transgenic calli.

All transformation experiments were performed in triplicate and the results pooled together.

The transformation vectors were introduced into cultured BMS cells using a silicon carbide fibre-mediated transformation technique as follows:

Preparation of silicon carbide whiskers

Dry whiskers were always handled in a fume cabinet, to prevent inhalation and possible lung damage. These whiskers may be carcinogenic as they have similar properties to asbestos. The Silar SC-9 whiskers were provided by the Advanced Composite Material Corporation Greer, South Carolina, USA. The sterile whisker suspensions were prepared in advance as follows. Approximately 50 mg of whiskers were deposited into a pre-weighed 1.5 ml Eppendorf tube, which was capped and reweighed to determine the weight of the whiskers. The cap of the tube was perforated with a syringe needle and covered with a double layer of aluminium foil. The tube was autoclaved (121 °C, 15 psi, for 20 minutes) and dried. Fresh whisker suspensions were prepared for each experiment, as it had been reported that the level of DNA transformation when using fresh suspensions was higher than that of older suspensions. A 5% (weight/volume) whisker suspension was prepared using sterile deionised water. This was vortexed for a few seconds to suspend the whiskers immediately before use.

DNA transformation into cells

All procedures were carried out in a laminar air flow cabinet under aseptic conditions. The DNA was transformed into the cells using the following approach. Specific modifications to this method are indicated in the text.

Cell and whisker suspensions were pipetted using cut down Gilson pipette tips. 100 µl of fresh BMS medium was measured into a sterile Eppendorf tube. To this was added 40 µl of the 5% (w/v) whisker suspension and 10 µl (1 mg/ml) of the plasmid DNA, which was vortexed at top speed for 60 seconds using a desktop vortex unit (Vortex Genie 2 Scientific Industries, Inc). Immediately after this period of vortexing, 500 µl of the cell suspension was added ie 250 µl of packed cells. The Eppendorf tube was then capped and vortexed at top speed for 60 seconds in an upright position. The same procedure was used to transform the other cell lines.

Results

As shown in Figure 8, all protease constructs resulted in a decrease in the number of calli regenerating on media containing bialophos, indicating that CPs might be detrimental to cell growth. pFPAT-B-Procaricain, that targets the CP precursor to mitochdria is as potent as barnase in the conditions of the experiment and totally prevents maize calli establishment. Similarly, the integration of pFPAT-Procaricain, pFPAT-Caricain and pFPAT-β-Caricain into the BMS cells genome leads to a reduction in the number of calli regenerating, with figures totalising 9.1%, 27.3% and 45.5% of the control respectively, 6 weeks after transformation.

The higher activity of the precursor-enzyme constructs compared to the mature enzymes is interesting since precursor proteins must loose their propeptide in order to be activated. However, caricain was specifically chosen for exemplification because its propeptide is very labile and a substantial proportion of it is thought to be cleaved in a self-catalytic reaction, even at neutral pH (Goodenough P W, personal communication). The lower activity of the mature enzyme constructs could be attributed to a folding problem since the amino-terminal propeptide is thought to act as a scaffolding that would allow the mature enzyme to take its correct conformation (Taylor *et al.*, 1995).

Although those results are preliminary, they have already been confirmed by a similar experiment undertaken on a wheat cell suspension system that can not be disclosed to date. All experiments are in the process to be repeated.

Example 2 - OVER-EXPRESSION OF CYSTEINE PROTEASE PROPEPTIDES IN CORN CALLI TO DEMONSTRATE THEIR POTENTIAL TO INHIBITTHE CORRESPONDING MATURE ENZYME

Aim

The objective of this experiment was to show that simultaneous expression of the protease and its cognate propeptide in cultured BMS corn cells results in an increased cell viability compared to the expression of the protease alone, as measured by the establishment of transgenic calli following transformation, thereby indicating that the expression of the propeptide is inhibiting the mature enzyme *in planta*.

Construction of the maize transformation vectors pFUN--Propeptide and pFUN-β-Propeptide

The 5'-end region of the caricain cDNA, encoding the propeptide area, was amplified by PCR on pET-WT-Procaricain using modified primers that introduce a *Bam*HI site at both ends of the new molecule. The PCR fragment was digested by *Bam*HI and subcloned into both the pFUN and the pFUN-β vectors also digested by *Bam*HI (between the polyubiquitin intron and the nos 3' terminator), to generate pFUN-Propeptide and pFUN-β-Propeptide respectively.

Transformation of BMS corn cells

The pFPAT-Caricain and pFPAT-Procaricain DNA vectors were respectively co-transformed into cultured BMS cells together with increasing amounts of the pFUN-Propeptide DNA vector. Similarly, the pFPAT-β-Caricain and pFPAT- β -Procaricain DNA vectors were respectively co-transformed into cultured BMS cells together with increasing amounts of the pFUN-β-Propeptide DNA vector. All transformations were performed using the silicon carbide fibre-mediated transformation technique described in Example 1. The

positive, negative and co-transformation controls described in Example 1 were also included in the experiment.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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CLAIMS

1. An isolated DNA construct comprising:-

a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism;

b) a second DNA sequence comprising a DNA sequence coding for a protease enzyme operably linked and under the control of the promoter sequence specified at (a);

whereby the presence or absence of the exogenous inducer or the activation of the developmental gene promoter specified at (a) results in expression of said protease enzyme.

2. A DNA construct comprising:-

a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism;

b) a second DNA sequence comprising a DNA sequence coding for a protease enzyme capable of disrupting cell function operably linked and under the control of the promoter sequence specified at (a);

c) a third DNA sequence comprising a promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of an organism; and

d) a fourth DNA sequence comprising a DNA sequence, the product of which is capable of inhibiting the protein specified in (b) operably linked and under the control of the promoter sequence specified at (c),

whereby the presence or absence of the exogenous inducer or the expression of the developmental gene promoter specified at (a) enables cell function to be disrupted and whereby the presence or absence of the exogenous inducer or the expression of

the developmental gene promoter specified at (c) prevents or reduces the disruption of cell function.

3. A DNA construct according to claim 2 wherein the first DNA sequence further comprises an operator sequence responsive to a repressor protein coded for by a DNA sequence comprised within a fifth DNA sequence which is operably linked and under the control of a sixth DNA sequence comprising an inducible promoter sequence, responsive to the presence or absence of an exogenous inducer, whereby expression of the repressor protein prevents or reduces the disruption of cell function.

4. A DNA construct according to claim 2 or claim 3 wherein the fourth DNA sequence comprises a DNA sequence coding for a member of a family of proteins which are naturally associated with the DNA sequence specified in (b).

5. A DNA construct according to claim 4 wherein the fourth DNA sequence comprises a DNA sequence coding for a protease propeptide, a protease inhibitor or an antibody directed to the protein capable of disrupting cell function.

6. A DNA construct according to any one of claims 1 to 5 wherein the protease is a targeted or non-targeted cysteine protease enzyme.

7. A DNA construct according to any one of the preceding claims wherein the developmental gene promoter is an early seedling/ seed promoter or a pathogen-induced promoter.

8. A DNA construct according to any one of the preceding claims capable of disrupting cell function in a plant cell or a mammalian cell.

9. Plant germplasm comprising a plant comprising a DNA construct of any one of claims 1 to 8.

10. A plant, plant seed or plant cell comprising a DNA construct of any one of claims 1 to 8.

11. A mammalian cell comprising a DNA construct of any one of claims 1 to 8 .

12. The use of a DNA construct as defined in any one of claims 1 to 8 to disrupt cell function.

13. DNA construct comprising:-

(a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of a plant; and

(b) a second DNA sequence comprising a DNA sequence, the product of which is capable of inhibiting a protein capable of controlling senescence in a plant operably linked and under the control of the promoter sequence specified at (a),

whereby the presence or absence of the exogenous inducer or the activity of the developmental gene promoter enables the synthesis of the protein capable of controlling senescence to be down-regulated or its activity to be substantially reduced, thereby enabling the process of senescence to be slowed down.

14. A DNA construct according to claim 13 wherein the second DNA sequence comprises a DNA sequence coding for a member of a family of proteins which are naturally associated with the protein capable of controlling senescence.

15. A DNA construct according to claim 13 or claim 14 wherein the second DNA sequence comprises a DNA sequence coding for a protease propeptide or a partial sense or antisense RNA.

16. A DNA construct according to any one of claims 13 to 17 wherein the protein capable of controlling senescence is a protease enzyme.

17. A DNA construct according to claim 15 or claim 16 wherein the protease is a targeted or non-targeted cysteine protease.

18. A DNA construct according to any one of claims 13 to 17 wherein the developmental gene promoter is a senescence-induced promoter.

19. A DNA construct according to any one of claims 13 to 18 capable of down-regulating or substantially reducing the activity of the protein capable of controlling senescence in a plant cell.

20. Plant germplasm comprising a plant comprising a DNA construct of any one of claims 1 to 19.

21. A plant, plant seed or plant cell comprising a DNA construct of any one of claims 1 to 19.

22. The use of a DNA construct as defined in any one of claims 13 to 19 to down-regulate the synthesis or substantially reduce the activity of a protein capable of controlling senescence.

23. A DNA construct comprising:-

(a) a first DNA sequence comprising either an inducible promoter sequence responsive to the presence or absence of an exogenous inducer or a developmental gene promoter capable of initiating gene expression in a selected tissue or at a selected stage of development of a plant; and

(b) second DNA sequence comprising a DNA sequence, the product of which is capable of down-regulating the synthesis or inhibiting a protein capable of controlling metabolism of stored protein in a plant operably linked and under the control of the promoter sequence specified at (a),

whereby the presence or absence of the exogenous inducer or the expression of the developmental gene promoter specified at (a) enables the synthesis of the protein capable of controlling metabolism of stored protein to be down-regulated or its activity to be substantially reduced, thereby altering the nature, the mobilisation and/or the distribution of stored products in plants.

24. A DNA construct according to claim 23 wherein the first DNA sequence further comprises an operator sequence responsive to a repressor protein coded for by a DNA sequence comprised within a third DNA sequence which is operably linked and under the control of an inducible promoter sequence, responsive to the presence of an exogenous inducer, coded for by a DNA sequence comprised within a fourth DNA sequence whereby expression of the repressor protein prevents alterations in the metabolism of stored proteins.

25. A DNA construct according to claim 23 or claim 24 further comprising a fifth DNA sequence comprising a DNA sequence coding for a heterologous protein which is operably linked and under the control of a sixth DNA sequence which comprises an inducible promoter, responsive to the presence or absence of an exogenous inducer, whereby expression of the heterologous protein gene prevents alterations in the metabolism of stored proteins.

26. A DNA construct according to any one of claims 23 to 25 wherein the protein capable of modifying the metabolism of stored protein in a plant is a protease enzyme.

27. A DNA construct according to claim 26 wherein the protein capable of modifying the metabolism of stored protein is a targeted or non-targeted cysteine protease enzyme.

28. A DNA construct according to any one of claims 23 to 27 wherein the second DNA sequence comprises a DNA sequence coding for a product which is naturally associated with the protein capable of modifying the metabolism of stored protein.

29. A DNA construct according to any one of claims 23 to 28 wherein the second DNA sequence comprises a DNA sequence coding for a protease propeptide.

30. A DNA construct according to any one of claims 23 to 29 wherein the second DNA sequence comprises a DNA sequence coding for a protease sense RNA or partial sense RNA.

31. A DNA construct according to any one of claims 23 to 29 wherein the second DNA sequence comprises a DNA sequence coding for a protease antisense RNA.

32. A DNA construct according to claim 30 or claim 31 wherein the second DNA sequence comprises a DNA sequence coding for a cysteine protease sense/ partial sense or antisense RNA.

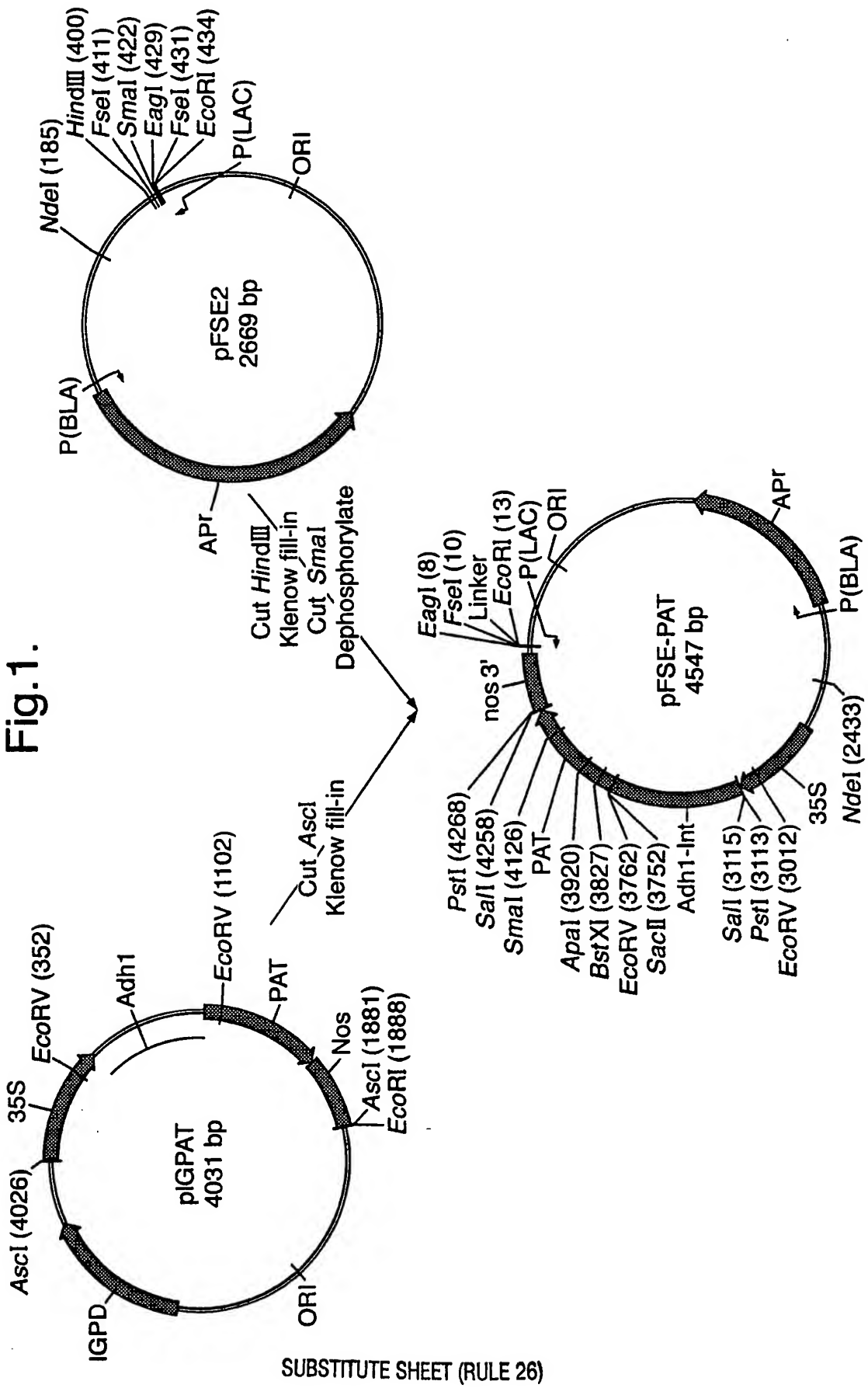
33. A DNA construct according to any one of claims 23 to 32 wherein the heterologous protein is a protease.

34. A DNA construct according to any one of claims 23 to 33 wherein the developmental gene promoter is an early seedling promoter.

35. A DNA construct according to any one of claims 23 to 34 capable of down-regulating the synthesis or substantially reducing the activity of the protein capable of modifying the metabolism of stored protein in plants.

36. A plant, plant seed or plant cell comprising a DNA construct of any one of claims 23 to 35

37. The use of a DNA construct as defined in any one of claims 23 to 35 to down-regulate the synthesis or substantially reduce the activity of a protein capable of modifying the metabolism of stored protein.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IGPD
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EcoRV (352)
Adh1
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EcoRI (1888)
ORI
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Klenow fill-in
pFSE2
2669 bp
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NdeI (185)
HindIII (400)
FseI (411)
SmaI (422)
EagI (429)
FseI (431)
EcoRI (434)
P(LAC)
ORI
APr
Cut HindIII
Klenow fill-in
Cut SmaI
Dephosphorylate
pFSE-PAT
4547 bp
EagI (8)
FseI (10)
Linker
EcoRI (13)
P(LAC)
ORI
APr
P(BLA)
nos 3'
PstI (4268)
SalI (4258)
SmaI (4126)
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ApaI (3920)
BstXI (3827)
EcoRV (3762)
SacII (3752)
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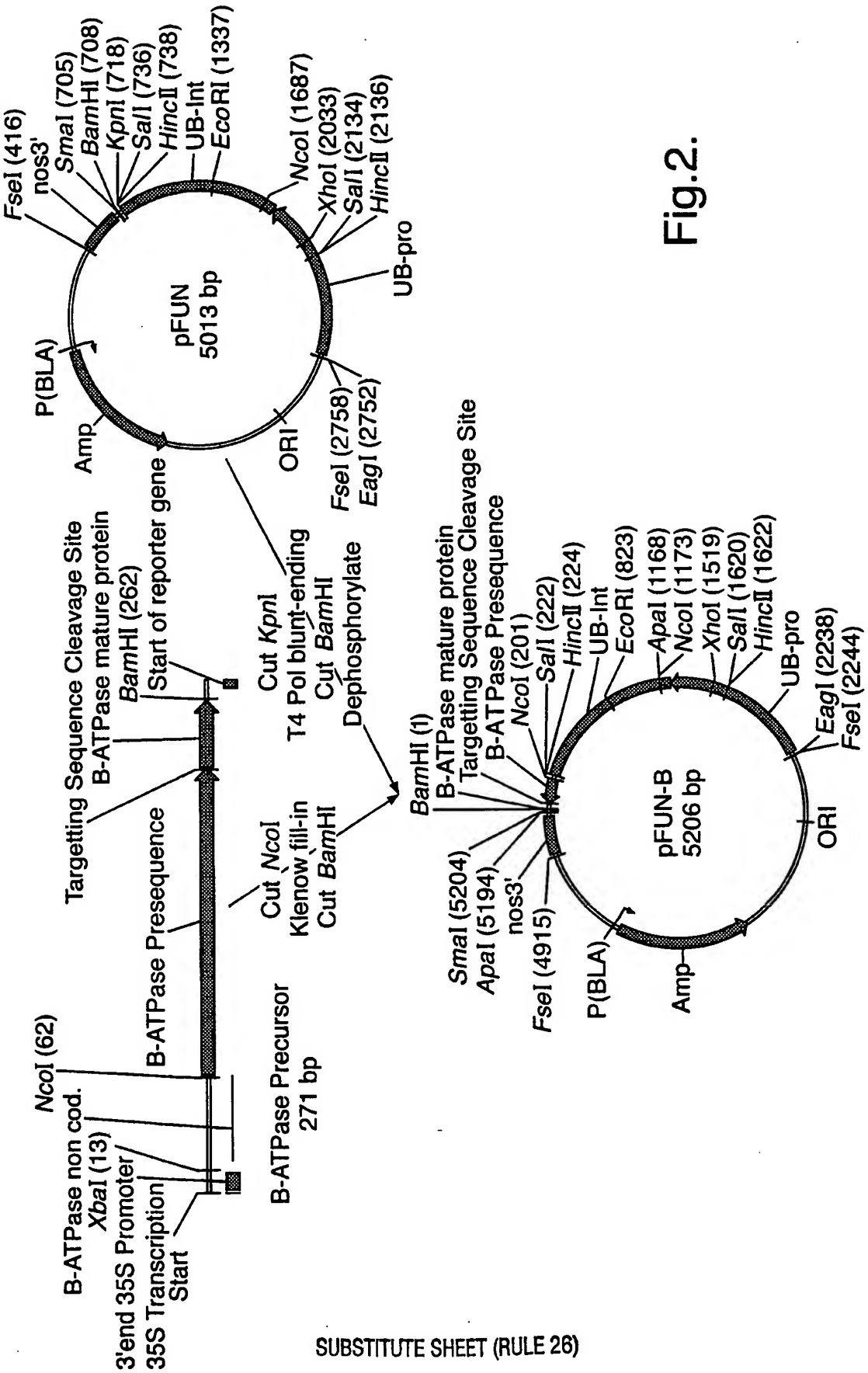
SalI (3115)
PstI (3113)
EcoRV (3012)
35S
NdeI (2433)

Fig.1.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SmaI (705)
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KpnI (718)
SalI (736)
HincII (738)
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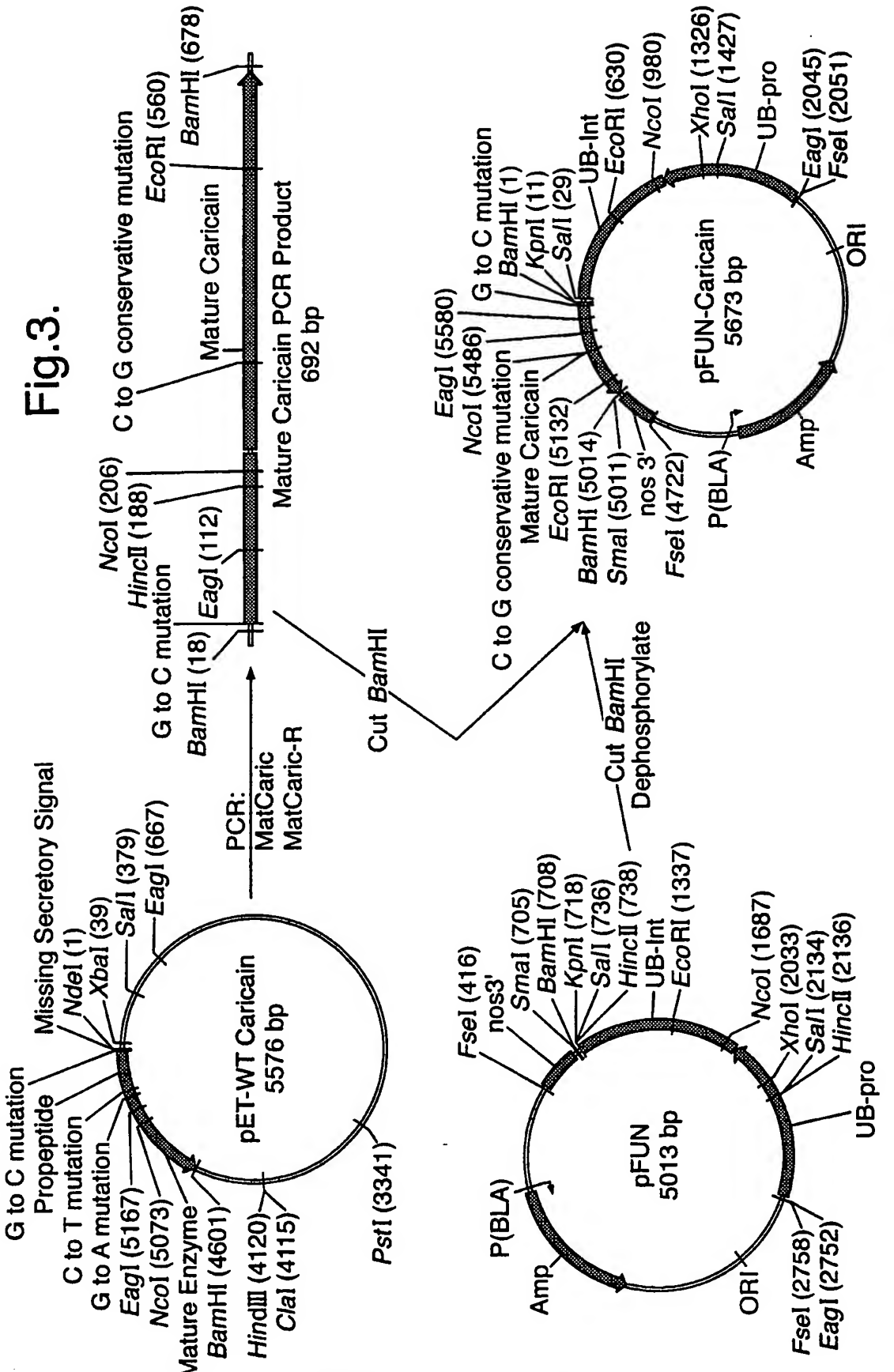
EcoRI (1337)
NcoI (1687)
XhoI (2033)
SalI (2134)
HincII (2136)
UB-pro
P(BLA)
Amp
pFUN
5013 bp
ORI
FseI (2758)
EagI (2752)
Cut KpnI
T4 Pol blunt-ending
Cut BamHI
Dephosphorylate
BamHI (1)
B-ATPase mature protein
Targetting Sequence Cleavage Site
B-ATPase Presequence
NcoI (201)
SalI (222)
HincII (224)
UB-Int
EcoRI (823)
ApaI (1168)
NcoI (1173)
XhoI (1519)
SalI (1620)
HincII (1622)
UB-pro
EagI (2238)
FseI (2244)
ORI
pFUN-B
5206 bp
P(BLA)
Amp
FseI (4915)
nos3'
ApaI (5194)
SmaI (5204)
Targetting Sequence Cleavage Site
B-ATPase mature protein
BamHI (262)
Start of reporter gene
Cut NcoI
Klenow fill-in
Cut BamHI
B-ATPase Presequence
B-ATPase Precursor
271 bp
NcoI (62)
B-ATPase non cod.
XbaI (13)
3'end 35S Promoter
35S Transcription
Start

Fig.2.

Fig.3.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Propeptide
C to T mutation
G to A mutation
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BamHI (4601)
HindIII (4120)
ClaI (4115)
PstI (3341)
Missing Secretory Signal
NdeI (1)
XbaI (39)
SalI (379)
EagI (667)
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MatCaric-R
BamHI (18)
G to C mutation
HincII (188)
NcoI (206)
EagI (112)
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C to G conservative mutation
EcoRI (560)
BamHI (678)
Mature Caricain PCR Product
692 bp
Cut BamHI
pFUN
5013 bp
P(BLA)
Amp
ORI
FseI (2758)
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FseI (416)
nos3'
SmaI (705)
BamHI (708)
KpnI (718)
SalI (736)
HincII (738)
UB-Int
EcoRI (1337)
NcoI (1687)
XhoI (2033)
SalI (2134)
HincII (2136)
UB-pro
Cut BamHI
Dephosphorylate
C to G conservative mutation
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NcoI (5486)
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EcoRI (5132)
BamHI (5014)
SmaI (5011)
nos 3'
FseI (4722)
P(BLA)
Amp
G to C mutation
BamHI (1)
KpnI (11)
SalI (29)
UB-Int
EcoRI (630)
NcoI (980)
XhoI (1326)
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FseI (2051)
ORI
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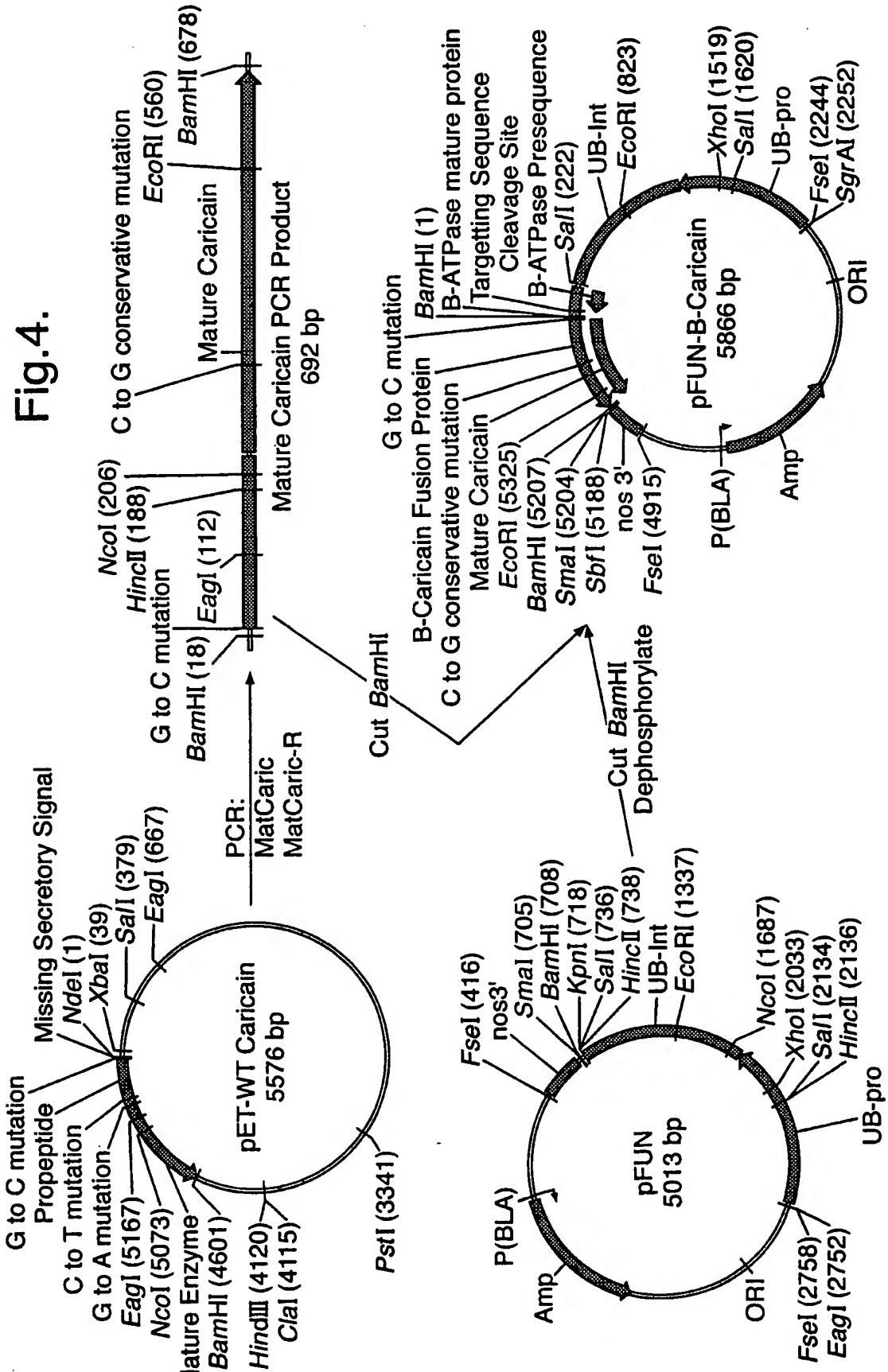
5673 bp

Fig.4.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Propeptide
C to T mutation
G to A mutation
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NcoI (5073)
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HindIII (4120)
ClaI (4115)
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5576 bp
PstI (3341)
Missing Secretory Signal
NdeI (1)
XbaI (39)
SalI (379)
EagI (667)
PCR:
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MatCaric-R
BamHI (18)
G to C mutation
HincII (188)
EagI (112)
NcoI (206)
C to G conservative mutation
EcoRI (560)
BamHI (678)
Mature Caricain
Mature Caricain PCR Product
692 bp
Cut BamHI
B-Caricain Fusion Protein
C to G conservative mutation
Mature Caricain
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BamHI (5207)
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SbfI (5188)
nos 3'
FseI (4915)
G to C mutation
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Targetting Sequence
Cleavage Site
B-ATPase Presequence
SalI (222)
UB-Int
EcoRI (823)
XhoI (1519)
SalI (1620)
UB-pro
FseI (2244)
SgrAI (2252)
ORI
Amp
P(BLA)
pFUN-B-Caricain
5866 bp
Cut BamHI
Dephosphorylate
FseI (416)
nos3'
SmaI (705)
BamHI (708)
KpnI (718)
SalI (736)
HincII (738)
UB-Int
EcoRI (1337)
NcoI (1687)
XhoI (2033)
SalI (2134)
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UB-pro
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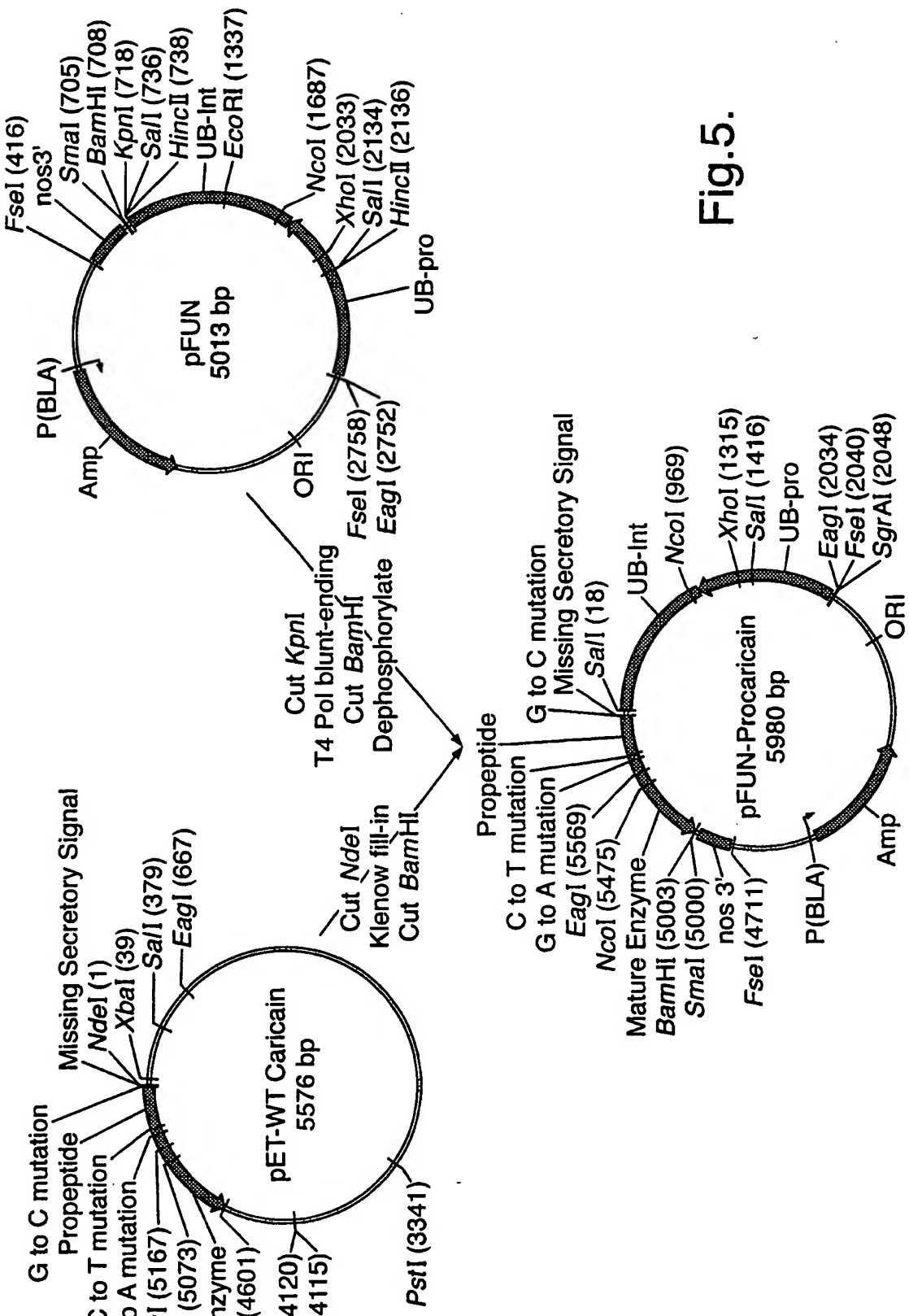
Amp
pFUN
5013 bp
ORI
FseI (2758)
EagI (2752)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5013 bp
P(BLA)
Amp
ORI
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T4 Pol blunt-ending
Cut BamHI
Dephosphorylate
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nos3'
SmaI (705)
BamHI (708)
KpnI (718)
SalI (736)
HincII (738)
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EcoRI (1337)
NcoI (1687)
XhoI (2033)
SalI (2134)
HincII (2136)
UB-pro
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5576 bp
Cut NdeI
Klenow fill-in
Cut BamHI
PstI (3341)
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Propeptide
C to T mutation
G to A mutation
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BamHI (4601)
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ORI
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FseI (2040)
SgrAI (2048)
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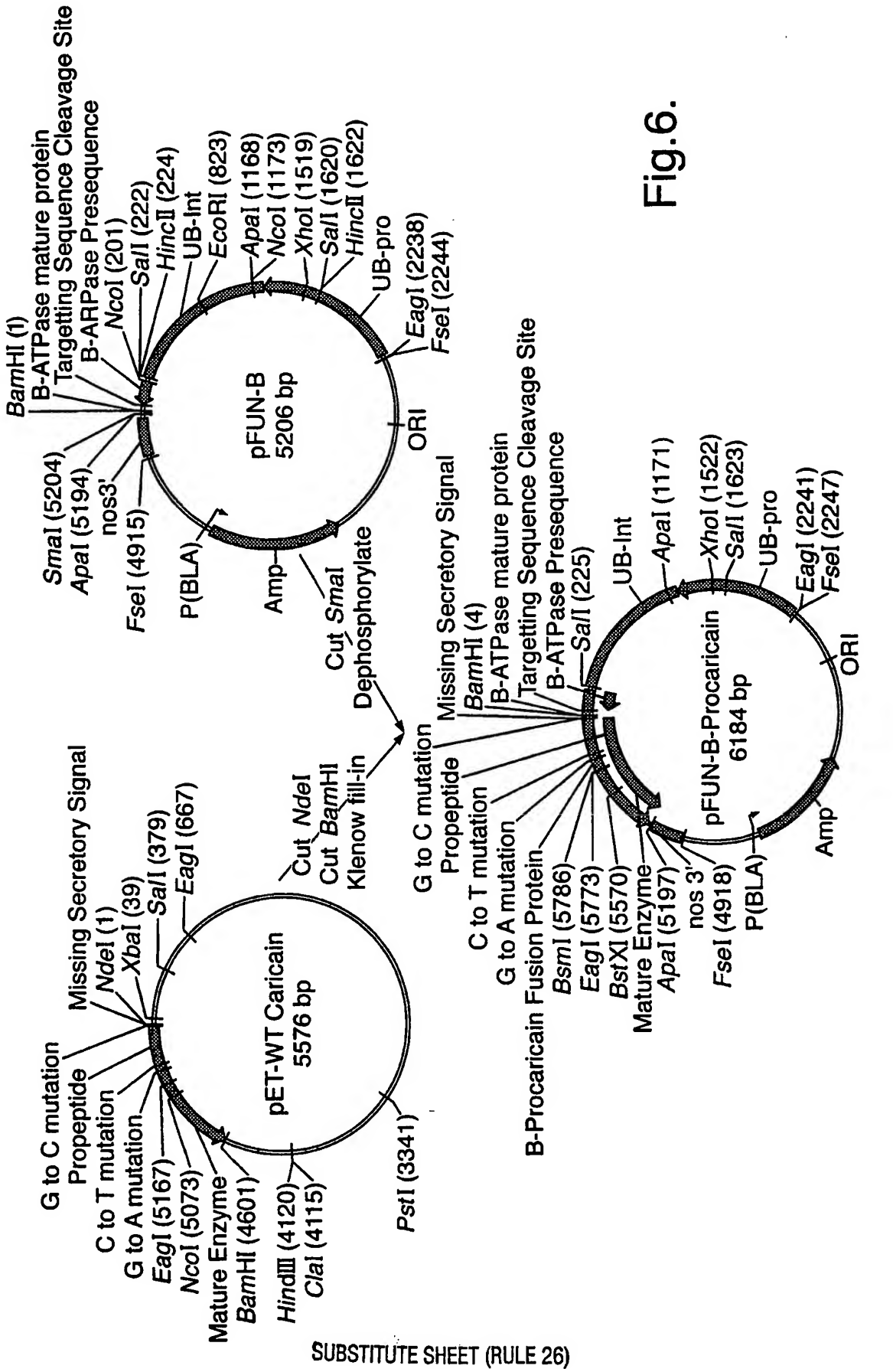
G to C mutation
Missing Secretory Signal
SalI (18)

Fig.5.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pFUN-B-Procaricain
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G to C mutation
Propeptide
C to T mutation
G to A mutation
Mature Enzyme
Missing Secretory Signal
Cut NdeI
Cut BamHI
Klenow fill-in
Cut SmaI
Dephosphorylate
B-Procaricain Fusion Protein
B-ATPase mature protein
Targetting Sequence Cleavage Site
B-ARPase Presequence
UB-Int
UB-pro
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Amp
ORI
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nos 3'
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NcoI (201)
SalI (222)
HincII (224)
EcoRI (823)
ApaI (1168)
NcoI (1173)
XhoI (1519)
SalI (1620)
HincII (1622)
EagI (2238)
FseI (2244)
NdeI (1)
XbaI (39)
SalI (379)
EagI (667)
PstI (3341)
ClaI (4115)
HindIII (4120)
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ApaI (1171)
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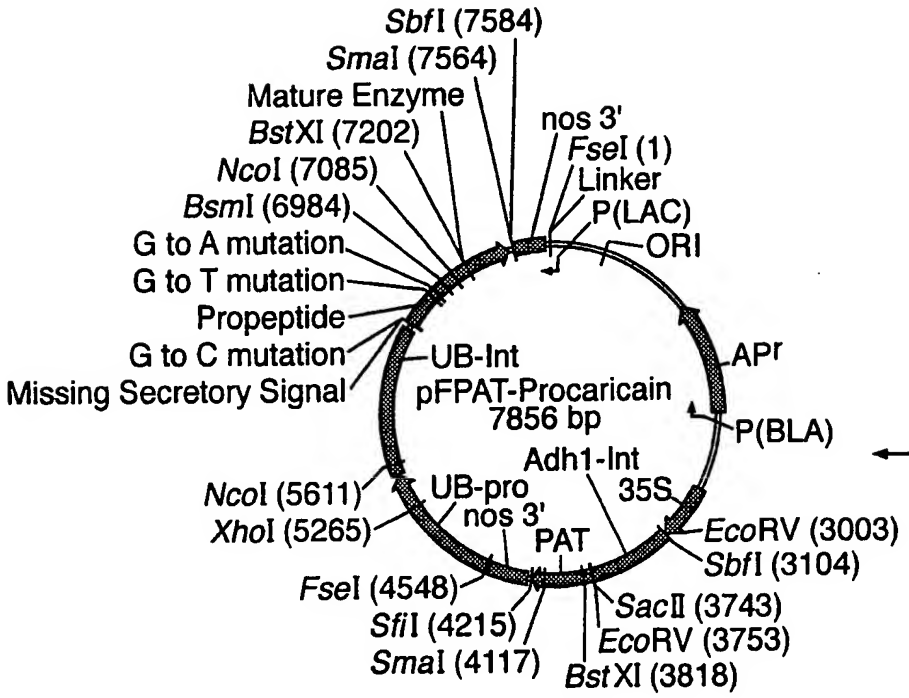
Fig.6.

Fig.7.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G to C mutation
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ORI
APr
P(BLA)
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SbfI (3104)
SacII (3743)
EcoRV (3753)
BstXI (3818)
NcoI (5611)
XhoI (5265)
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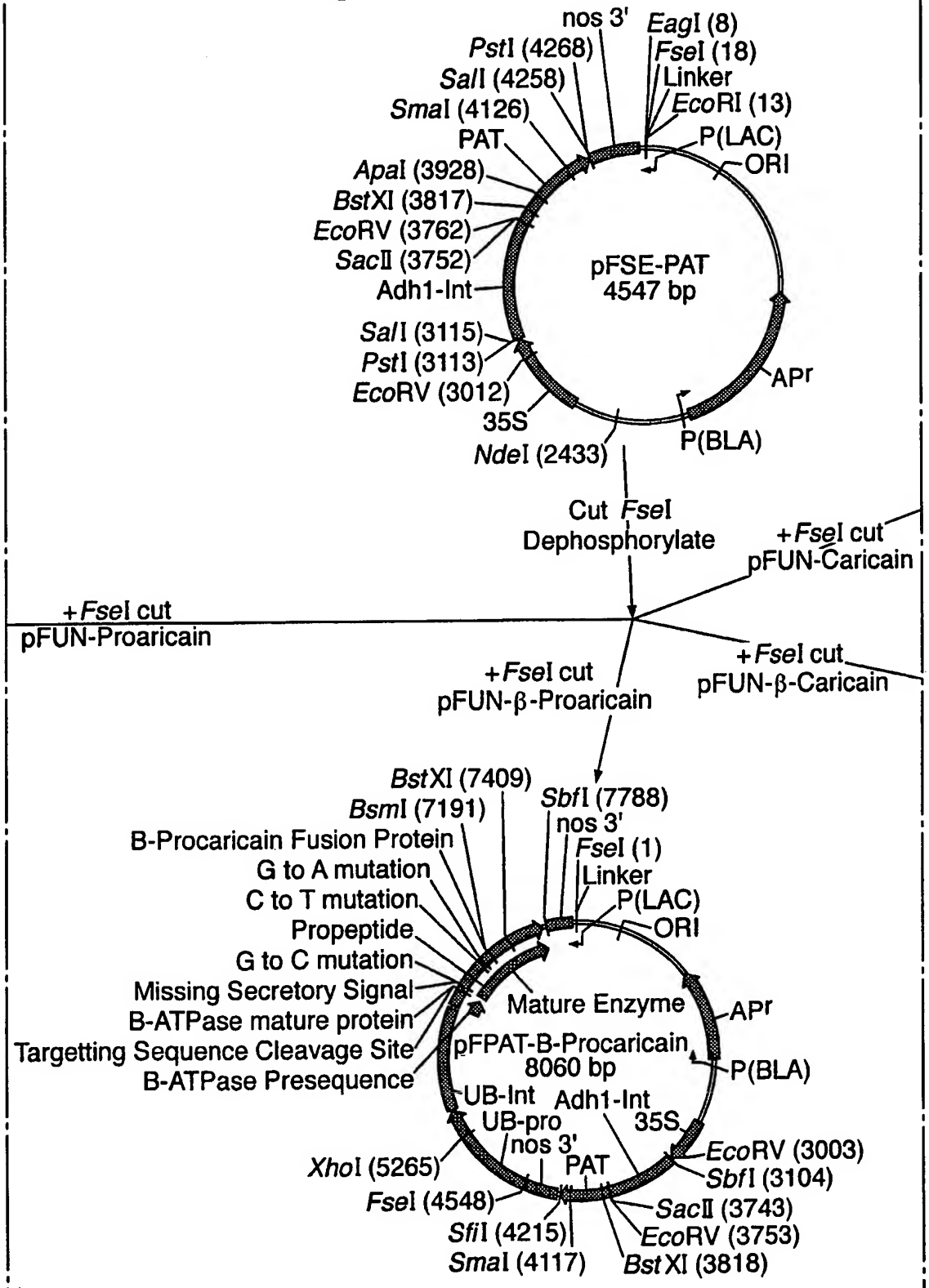
SmaI (4117)

Fig.7 (Cont i).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SalI (4258)
Linker
SmaI (4126)
EcoRI (13)
PAT
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ApaI (3928)
ORI
BstXI (3817)
EcoRV (3762)
SacII (3752)
pFSE-PAT
4547 bp
Adh1-Int
SalI (3115)
PstI (3113)
APr
EcoRV (3012)
35S
P(BLA)
NdeI (2433)
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Dephosphorylate
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pFUN-Caricain
+FseI cut
pFUN-Proaricain
+FseI cut
pFUN-β-Caricain
+FseI cut
pFUN-β-Proaricain
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SbfI (7788)
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C to T mutation
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Propeptide
ORI
G to C mutation
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Mature Enzyme
APr
B-ATPase mature protein
pFPAT-B-Procaricain
8060 bp
Targetting Sequence Cleavage Site
P(BLA)
B-ATPase Presequence
UB-Int
Adh1-Int
UB-pro
35S
nos 3'
EcoRV (3003)
XhoI (5265)
PAT
SbfI (3104)
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EcoRV (3753)
SmaI (4117)
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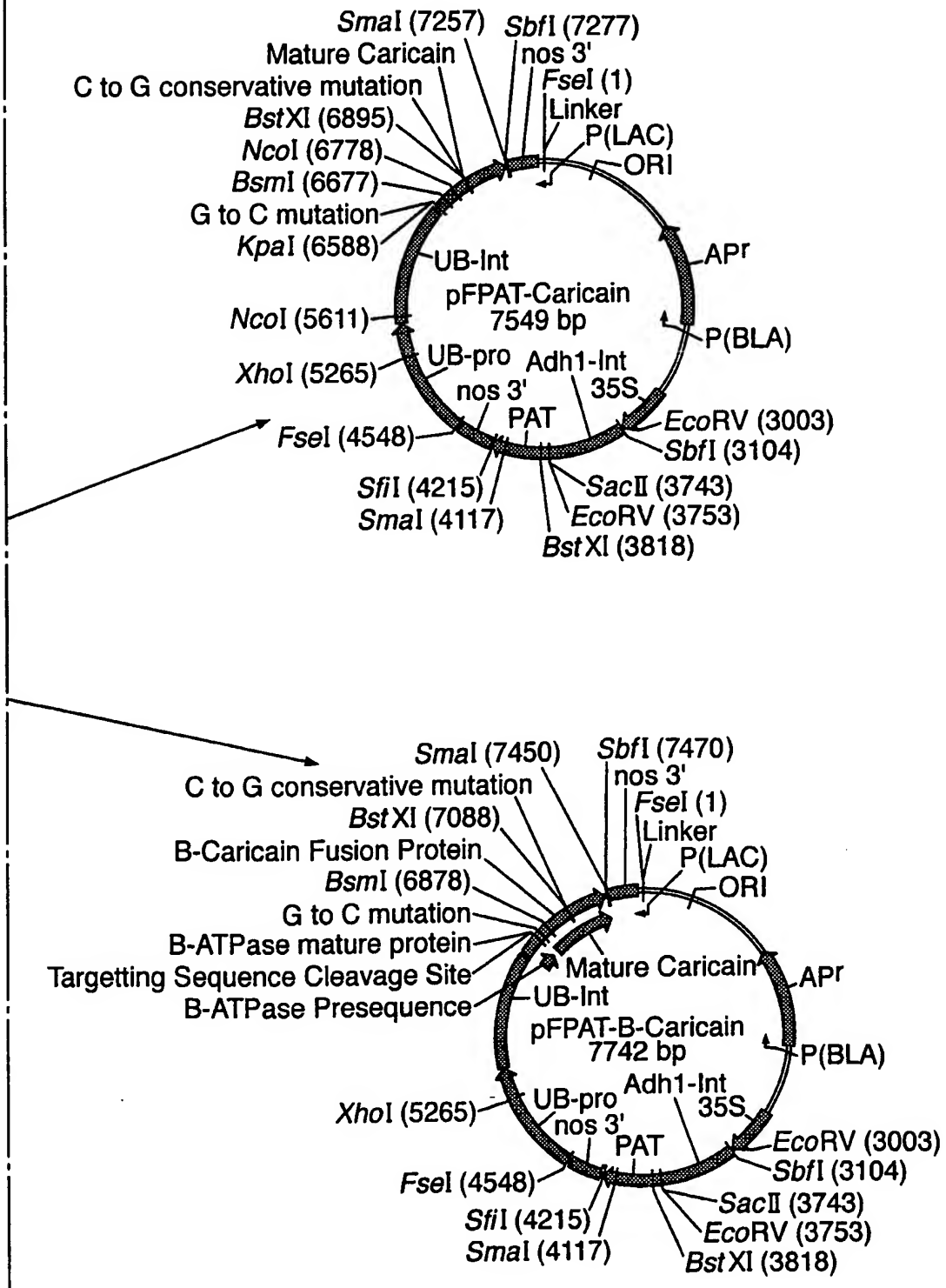
Fig.7 (Cont ii).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UB-pro
Adh1-Int
XhoI (5265)
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PAT
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SbfI (3104)
SfiI (4215)
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BstXI (7088)
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BsmI (6878)
ORI
G to C mutation
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Mature Caricain
APr
Targetting Sequence Cleavage Site
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B-ATPase Presequence
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Adh1-Int
UB-pro
35S
XhoI (5265)
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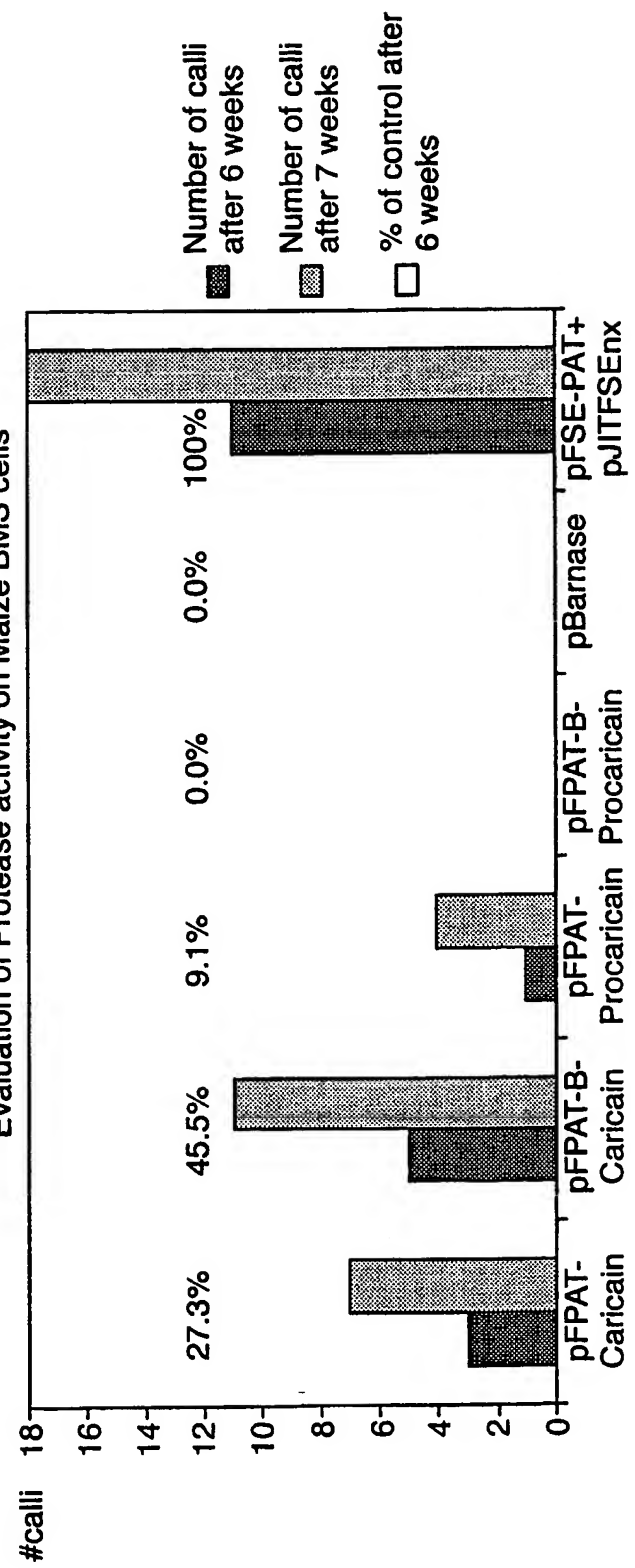
Fig.8.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#calli
0
2
4
6
8
10
12
14
16
18
27.3%
45.5%
9.1%
0.0%
0.0%
100%
pFPAT-Caricain
pFPAT-B-Caricain
pFPAT-Procaricain
pFPAT-B-Procaricain
pBarnase
pFSE-PAT+ pJITFSEnx
Number of calli after 6 weeks
Number of calli after 7 weeks
% of control after 6 weeks

Intern. al Application No
PCT/GB 99/02699

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/57 C12N15/82 C12N15/11 C12N5/10 A01H5/00
A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35983 A (ZENECA LTD ;JEPSON IAN (GB); GREENLAND ANDREW JAMES (GB); THOMAS D) 2 October 1997 (1997-10-02) cited in the application	1-14, 18-25, 28,34-37
Y	see the whole document; esp. pp. 2-12	5

X	WO 97 44465 A (MONSANTO CO) 27 November 1997 (1997-11-27)	23-25, 28,34-37
Y	the whole document	29

X	WO 95 07993 A (ZENECA LTD ;SMART CATHERINE MARGARET (GB); THOMAS HOWARD (GB); HOS) 23 March 1995 (1995-03-23) cited in the application	13-22
Y	see the whole document; esp. p.25(3); examples 3 ff.	15

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 1999	15/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 04393 A (US GOVERNMENT ;DELTA & PINE LAND CO (US)) 15 February 1996 (1996-02-15) the whole document	1,8-10, 12,13, 19-21, 23,24, 34-37
X	WO 94 03619 A (BRIGHT SIMON WILLIAM JONATHAN ;PAINE JACQUELINE ANN MARY (GB); JEP) 17 February 1994 (1994-02-17) the whole document	1,8-10, 12,20,21
X	EP 0 412 006 A (PLANT GENETIC SYSTEMS NV) 6 February 1991 (1991-02-06) the whole document	1,8-10, 12,20,21
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INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/GB 99/02699

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims 1-12 completely; 20, 21 partially

DNA constructs comprising an inducible promoter or a developmental gene promoter driving the expression of a protease, preferably a cysteine protease, capable of disrupting cell function. Said constructs optionally comprising a second promoter driving a restorer function, said restorer optionally being a protease propetide.
Further embodiments as formulated in the claims.
Plant gemplasm, plants, plant cells, and seeds comprising said constructs.
The use of said constructs to disrupt cell function.

2. Claims 13-19, 22-37 completely; 20, 21 partially

DNA constructs comprising an inducible promoter or a developmental gene promoter driving the expression of a DNA sequence the product of which is capable of inhibiting a protein capable of controlling either senescence, or metabolism of stored proteins, preferably a protease inhibiting product, more preferably a protease propeptide.
Said constructs optionally comprising a second promoter driving a repressor function inhibiting said first promoter, or optionally comprising a third promoter driving the expression of a heterologous restorer, said restorer optionally being a protease. Further embodiments as formulated in the claims.
Plant gemplasm, plants, plant cells, and seeds comprising said constructs.
The use of said constructs to downregulate the synthesis or reduce the activity of a protein capable of controlling senescence or modifying the metabolism of stored protein.

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PCT/GB 99/02699

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